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Over-expression of mouse ornithine decarboxylase gene under the control of fruit-specific promoter enhances fruit quality in tomato

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Abstract Diamine putrescine (Put) and polyamines; spermidine (Spd) and spermine (Spm) are essential component of every cell because of their involvement in the regulation of cell division, growth and development. The aim of this study is to enhance the levels of Put during fruit development and see its implications in ripening and quality of tomato fruits. Transgenic tomato plants over-expressing mouse ornithine decarboxylase gene under the control of fruit-specific promoter (2A11) were developed. Transgenic fruits exhibited enhanced levels of Put, Spd and Spm, with a concomitant reduction in ethylene levels, rate of respiration and physiological loss of water. Consequently such fruits displayed significant delay of on-vine ripening and prolonged shelf life over untransformed fruits. The activation of Put biosynthetic pathway at the onset of ripening in transgenic fruits is also consistent with the improvement of qualitative traits such as total soluble solids, titratable acids and total sugars. Such changes were associated with alteration in expression pattern of ripening specific genes. Transgenic fruits were also fortified with important nutraceuticals like lycopene, ascorbate and antioxidants. Therefore, these transgenic tomatoes would be useful for the improvement of tomato cultivars through breeding approaches.

Keywords Tomato · Ornithine decarboxylase · Polyamines · Ethylene · Fruit ripening · Transgenic plants

Introduction

Ripening in climacteric fruits is regarded as necessary evil wherein it imparts desirable flavor, colour and texture to the fruit (Giovannoni 2001). Desptite enhancing the fruit quality, ripening causes considerable post-harvest losses due to excessive softening and over-ripening (Payasi and Sanwal 2010). Several attempts have been made to introduce delayed ripening trait in climacteric fruits, and the most widely used strategies are the down-regulation of ripening induced genes, including ethylene metabolism, modulation of fruit softening or cell wall metabolism (Hamilton et al. 1990; Oeller et al. 1991; Lashbrook et al. 1998; Brummell et al. 1999; Wang et al. 2005; Xie et al. 2014). However, these strategies have certain limitations. For instance, the manipulation of ethylene pathway resulted in delayed ripening however, overall fruit quality was also affected (Xiong et al. 2005). Moreover, most of these strategies suffered a setback due to the functional redundancy shown by other members of such gene families (Gupta et al. 2013).

Diamine putrescine (Put) and polyamines (PAs); spermidine (Spd) and spermine (Spm) are omnipresent polycationic small molecules that are present in all living cells. They regulate a plethora of biological processes, including gene expression and cell signalling, and are absolutely essential for normal growth and development of various

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organisms (Alcázar et al. 2010; Igarashi and Kashiwagi 2010; Kusano et al. 2008; Schwartz et al. 2011; Takahashi and Kakehi 2010). PA biosynthesis pathway initiates with the production of diamine Put, an obligate precursor of higher PAs, Spd and Spm, directly by decarboxylation of ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17). In addition to the ODC pathway, plants and bacteria have an alternate pathway for Put biogenesis, which involve decarboxylation of arginine by arginine decarboxylase (ADC; EC 4.1.1.19). Spd and Spm are synthesized from Put by the sequential addition of amino propyl groups, and these reactions are catalyzed by Spd and Spm synthases (SPDS/SPMS/; EC 2.5.1.16/EC2.5.1.22) respectively. The amino propyl groups are donated by the decarboxylated S-adenosylmethionine (SAM), which in turn is produced from SAM by SAM decarboxylase (SAMDC; EC 2.5.1.1.6) (Sauter et al. 2013).

PAs are implicated in early fruit development and ripening. They act as anti-ripening and anti-senescence agents, by antagonizing ethylene action (a ripening hormone) in fruit and vegetative tissues (Cassol and Mattoo 2002). The exogenous treatment of PAs in various fruits has been shown to retard fruit colour change, respiration rate and senescence, delay ethylene emission and fruit ripening, increase fruit firmness and cell wall stability and reduced mechanical stress and chilling symptoms (Apelbaum et al. 1981; Valero et al. 2002). PAs, along with salicylic acid also regulate ethylene biosynthesis by influencing *ACC synthase* transcript accumulation (Li et al. 1992; Handa and Mattoo 2010). PAs and ethylene affect plant growth and senescence antagonistically by sharing SAM as a precursor, where a plant cell can divert SAM pools either into PA biosynthesis, ethylene biosynthesis, or both (Kumar and Rajam 2004; Lasanajak et al. 2014). Tomato transgenics over-expressing yeast *SAMDC* and yeast Spd synthase (*SPDS*) showed enhanced lycopene content, fruit juice viscosity and ethylene levels along with the increased Spd and Spm production (Mehta et al. 2002; Nambeesan et al. 2010). The fruits of these transgenics accumulating higher levels of Spd and Spm also exhibited quantitative changes in transcript profile of ethylene receptor populations and component(s) of the ethylene signalling pathway. These studies suggest that PA-ethylene nexus plays a crucial role in fruit ripening. Therefore, while looking at multifunctional and regulatory aspects of PAs and ethylene, it is possible that controlled manipulation of these key regulators rather than a structural or a regulatory gene operating in single branch of the biosynthesis pathway may result in better improvement of fruit shelf life and quality traits. Hence the utilization of the metabolic interactions of pathways related to post-harvest characteristics like the competitive interaction of PAs with that of ethylene seems to be an alternative strategy for the modulation of fruit ripening and quality. Accordingly, PA biosynthetic enzymes qualify as targets to control progression of ripening in various fruits. In this regard, most of the studies have exploited the higher PAs (Spd and Spm), but little has been reported for the utilization of Put. Interestingly, the three PAs have been shown to influence fruit ripening differentially, where the effects of diamine Put generally contrast with that of Spd and Spm (Handa and Mattoo 2010). This warrants further investigation to demonstrate the role of individual PAs in fruit ripening. Therefore, in this study, we over-expressed Put biosynthesis gene, *ODC* in tomato under the control of fruit-specific promoter (2A11) to demonstrate its role in ripening.

Results and discussion

Development of homozygous transgenic tomato plants over-expressing ODC transgene

The selection of *ODC* gene source i.e., mouse was based on the availability of gene sequence from said source at the commencement of study and also because of its least homology (at the nucleotide level, ~45 %) to its tomato counterpart. Several independent tomato transformants were developed using 2A11-*ODC* gene construct. Initial screening of primary transformants (T_0) by PCR using primers specific to *NPT*-II and m-*ODC* genes revealed the presence of the transgenes. DNA blot analysis of T_0 plants detailed the copy number of transgene, which varied from one to multiple in different events. Out of seven lines analyzed, four lines (LeODC22, LeODC23, LeODC27 and LeODC32) showed single copy insertion while rest of the lines (LeODC30 and LeODC44) showed multiple copy insertion (Supplementary Fig. 2). In order to negate the possibility of differential expression due to variable copy number (Hobbs et al. 1993), single copy lines (also confirmed as homozygous lines) were utilized in the study. The results of segregation analysis conferred that LeODC22, LeODC23, LeODC27 and LeODC32 lines as homozygous for *ODC* transgene integration (Supplementary Fig. 2). The morphology of transgenic plants was normal. They were fertile with pollen viability ranging from 90 to 95 %, which is similar to that of untransformed plants (UT) (Supplementary Fig. 4). The onset of ripening in transgenic fruits was at par with the UT fruits (data not shown).

Stability of m-*ODC* gene expression across generations

Northern hybridization studies showed strong fruit-specific m-*ODC* transgene expression across four generations of transgenic tomato (i.e. T_0–T_3). m-*ODC* transcripts were first noticed at the mature green (MG) stage and their titers increased during breaker (BR) and pink red (PR) stage

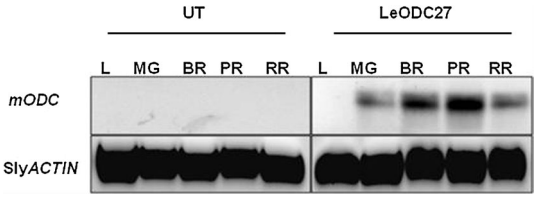

Fig. 1 Stable expression of transgene across four generations (T_0–T_3) of LeODC transgenic tomato restricted to ripening fruits. Northern blot analysis was done using radio-labeled m-*ODC* gene fragment to probe transgene transcript accumulation in T_3 generation fruits. Radio-labeled Sl*ACTIN* gene probe was used to ascertain *ACTIN* gene levels as internal control. *MG* mature green, *BR* breaker, *PR* pink red, *RR* red ripe, stages of fruit ripening

followed by a sharp decline at red ripe (RR) stage (Fig. 1). Transcript accumulation was concomitant with the activity of fruit-specific promoter (2A11). This promoter has been very well characterized from tomato plant, where 2A11 gene expresses strictly in a fruit-specific manner (Pear et al. 1989).

Polyamine levels during different stages of fruit ripening

The fruits of UT tomato plants showed maximum PA titres in MG tissue, followed by a steep fall till RR stage. In contrast to the free and conjugated fractions, the bound fraction was present at relatively low level in UT fruits (Fig. 2, Supplementary File 1). Put and Spd concentrations declined during ripening with the Spm being in very low levels and negligible at final stage. As expected, transgenic fruits exhibited an overall increase in PAs (up to 2.0 fold) over UT fruits, with significant increase in Put (1.6–1.9 fold) due to the over-expression of *ODC* transgene. The increased Put accumulation was also accompanied by concomitant increase in higher PAs, viz., Spd (1.5-fold) and Spm (1.5–1.85 fold). In fruits of LeODC transgenics, high concentration of PAs were recorded at BR stage and thereafter, PA levels subsided although the concentrations were still significantly high levels as compared to UT fruits. The free (2.0 fold) and bound (1.5 fold) fractions contributed majorly to the enhanced PA pools as compared to conjugated fraction (Fig. 2a, Supplementary File 1). There was no significant change in mRNA levels of native tomato *ODC* and *ADC* genes, but *SAMDC*1 and *SPDSYN* transcripts increased in transgenic fruits over UT fruits (Fig. 2b, Supplementary Fig. 5a). In fact, *SAMDC*1 has been suggested to be predominantly responsible for SAMDC activity in ripening fruits (Gupta et al. 2013). Put upsurge in transgenic fruits is mainly because of the over-expression of m-*ODC* gene, while increased *SAMDC1* and *SPDSYN* transcripts is accountable for the enhanced Spd and Spm content in transgenic fruits. These results demonstrated that the over-expression of heterologous *ODC* in tomato fruits has led to the up-regulation of the whole PA biosynthetic pathway and resulted in PA accumulation. Similar observations were obtained in transgenic wheat plants expressing an oat *ADC* (Bassie et al. 2007). In an another study, Put has been shown to enhance the conversion of precursor SAMDC into active SAMDC, leading to increased amounts of active enzyme and caused the activation of the PA biosynthesis (Kameji and Pegg 1987).

Ethylene production and expression profile of ethylene biosynthesis genes

Ethylene holds the well established status of a ripening inducer and stimulator in climacteric fruits. While it accelerates different pathways needed for completion of ripening, it also adversely affects the post-harvest storage of fruits. Our results showed that ethylene biosynthesis was significantly impaired in LeODC transgenic fruits. There was ~40 % reduction in ethylene levels in transgenic fruits over UT fruits (Fig. 3a). Further, fruit-specific homologues of 1-aminocyclopropane-1-carboxylate synthase (*ACS*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) genes displayed transcript reduction in transgenic fruits (Fig. 3b, Supplementary Fig. 5b) and this has resulted in low levels of ethylene in transgenic fruits over UT fruits. There are several studies, which reported the negative correlation between PA levels and *ACS* and *ACO* expression. For instance, PAs have been shown to inhibit *ACS* transcript accumulation (Handa and Mattoo 2010; Li et al. 1992; Ziosi et al. 2006). PAs and ethylene intersect biosynthetically at a common point for precursor, SAM (Winer and Apelbaum 1986). Thus, an inverse relationship exists between PAs and ethylene biosynthesis during fruit ripening which is modulated by changes in native SAMDC activity. Therefore, in the present study, the over-production of Put and its conversion into Spd and Spm might have resulted in limiting SAM flux towards ethylene biosynthesis. A recent report by Lasanajak et al. (2014) further substantiates our results. Further, these results are also consistent with results obtained with the use of PA inhibitors (Kushad and Dumbroff 1991; Parra-Lobato and Gomez-Jimenez 2011) and transgenic approaches employed for the fruit quality improvement (Kumar et al. 1996; Lasanajak et al. 2014; Madhulatha et al. 2014). However, Mehta et al. (2002) reported elevated ethylene levels in tomato transgenics over-expressing yeast *SAMDC* gene, and suggested that the SAM is not a limiting factor for polyamine and ethylene biosynthesis.

Improvement in storage attributes of transgenic fruits

Data obtained from four generations, i.e. T_0–T_3 of transgenic fruits consistently showed decreased rate of respiration (CO_2 levels), reduced physiological loss of water

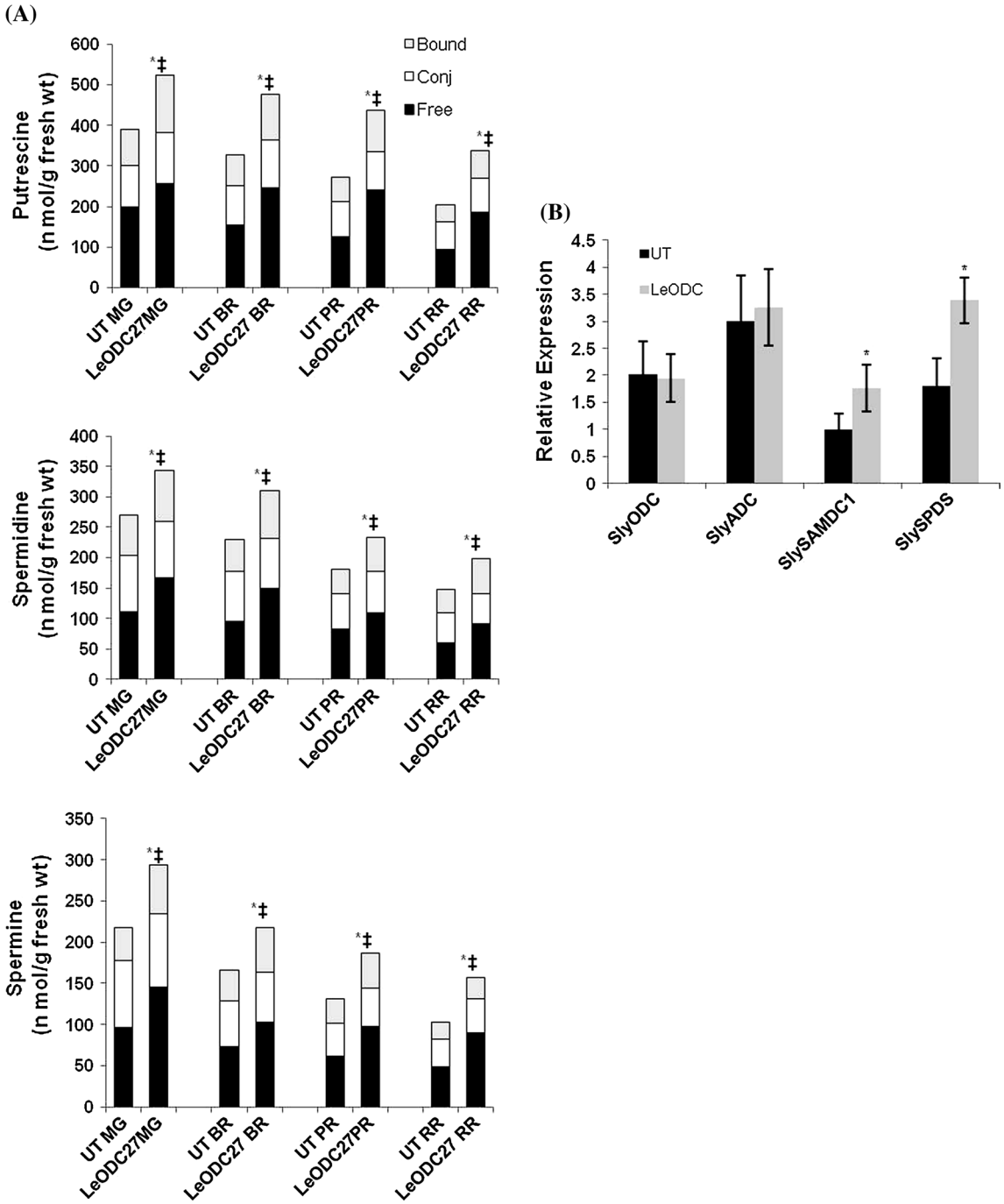

Fig. 2 PA levels in transgenic and UT fruits at MG, BR, PR and RR stages in T_3 generation. PA estimation was done thrice with tissue extract from third generation of UT and transgenic fruits, in 10 % PCA by TLC method. Three biological replicates were maintained in the experiment ($n = 3 \times 3 = 9$). † and ‡ denote significant differences in free and bound fractions respectively from UT at 5 % level. **a** Put, Spd and Spm levels in transgenic and UT fruits. **b** Relative expression levels of PA biosynthesis genes in UT and T_3 transgenic fruits. Relative quantification was performed by qRT-PCR using comparative D cycle threshold (CT) method. Expression levels of all the genes were normalized to SlyActin levels. The *data* represent the average of three biological replicates, each with two technical replicates and was plotted with error bars showing standard deviation. *Significant at $p \geq 0.05$ over UT control. Expression analysis was done with three biological replicates (sample size $= 3 \times 2$)

(PLW), delayed on-vine ripening and extended shelf life (Fig. 4). LeODC transgenic fruits recorded 15–40 % reduction in CO_2 evolution when compared to the UT ones (Fig. 4b). However, LeODC22 and LeODC32 did not show reduction in respiratory activity, which could be attributed to the position effect of transgene integration. PLW was

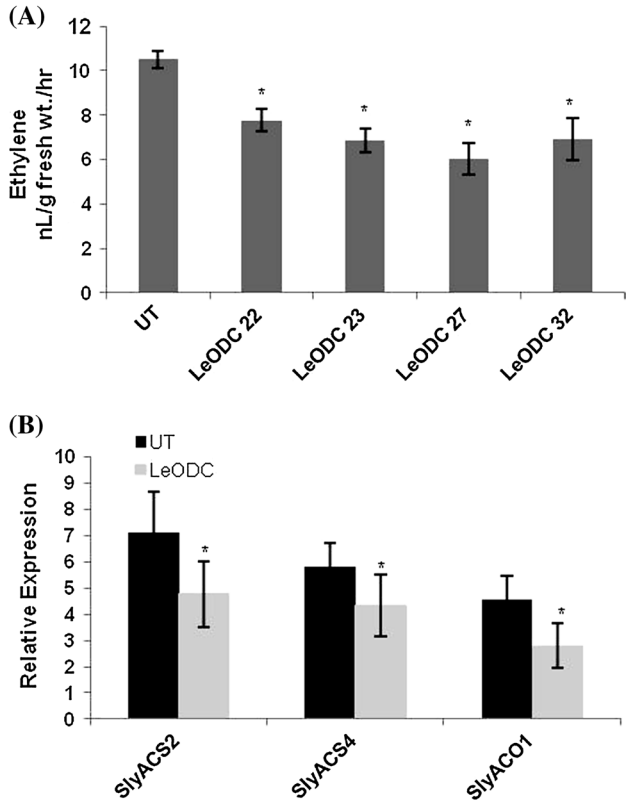

Fig. 3 Ethylene levels in fruits of UT and transgenic tomato plants. **a** Ethylene evolution was measured in UT and transgenic fruits (RR) through GC, in four generations (T_0–T_3). Data shown is the representation of mean of nine biological replicates in each of the ten independent experiments from T_3 generation ($n = 9 \times 10 = 90$). **b** Relative expression levels of ethylene biosynthesis genes in UT and transgenic fruits. Relative quantification was performed by qRT-PCR using comparative D cycle threshold (CT) method. Expression levels of all the genes were normalized to SlyActin levels. The data represent the average of three biological replicates, each with two technical replicates and was *plotted with error bars* showing standard deviation. *Significant at $p \geq 0.05$ over UT control. Expression analysis was done in T_3 generation with three biological replicates (sample size $= 3 \times 2$)

also slowed down in transgenic fruits (Fig. 4c). There was ~7 days delay in on-vine transition from BR to RR stage as compared to UT fruits (Fig. 4a). Skin wrinkles were seen in UT fruits after 6–7 days whereas transgenic fruits started showing sign of senescence after 10–15 days of storage at room temperature (Fig. 4d). Fruits kept at 4 °C at RR stage showed approximately 20–25 days of shelf life without showing chilling symptoms, whereas UT fruits started rotting after 10–12 days. Therefore, transgenic fruits showed an extended shelf life of ~7 days at room temperature and ~15 days at 4 °C as compared to UT fruits. Transgenic fruits (RR) were also found to be firmer than UT fruits (Data not shown).

Put treatments in different fruits have shown to reduce their respiration rate by hampering ethylene production (Khan et al. 2008; Serrano et al. 2003; Torrigiani et al. 2012). Decline in respiratory activity and PLW in transgenic fruits supports for the delayed on-vine and extended shelf life. The prolonged shelf life is also attributed to low ethylene levels in transgenic tomato fruits since the ethylene levels in harvested tomato fruit bears a negative correlation with its shelf life (Guillén et al. 2007).

The textural softening during fruit ripening results from separation and dissolution of cell wall components. Consequently cell wall stability and rigidity majorly determine the processing characteristics, firmness and quality of tomato fruits. Various cell wall depolymerizing enzymes like polysaccharide hydrolases/glycoside hydrolase, transglycosylases, lyases and expansins get induced during ripening and are responsible for ripening related firmness loss (Brummell 2006). The expansin 1 (*EXP1*), β-galactosidase 4 (*TBG4*), polygalacturonase (*PG*) and xyloglucan endotransglycosylase/hydrolases 5 (*XTH5*) genes have been shown to be specifically expressed during fruit ripening and play major role in fruit softening (Pirrello et al. 2009). Expression analysis of both *EXP1* and *TBG4* revealed elevated transcript levels in transgenic fruits over UT fruits (Fig. 4e, Supplementary Fig. 5c). *PG* showed no difference in transcript abundance but considerable reduction was observed in *XTH5* expression in transgenic fruits over UT fruits (Fig. 4e, Supplementary Fig. 5c).

Although our findings on expression of cell wall loosening genes are in conflict with the data on enhanced shelf life of fruits, the increase in bound PA fractions in transgenic fruits seems to be important for the enhanced shelf life possibly by stabilizing the membranes. Put, Spd and Spm are covalently associated with pectic polysaccharides in plant cell walls (D'Orazi and Bagni 1987). The interference with PA biosynthesis has been shown to hamper cell wall formation making it amorphous, while the exogenous treatment of PAs can reverse these changes (Berta et al. 1997). Knee and Bartley (1981) have demonstrated that SAM methylation of the free carboxylic groups in pectin leads to disruption of calcium (Ca) cross linkages of adjacent polyuronides, which causes loss in cohesion of cell walls. The negatively charged carboxyl groups (COO–) of uronic acids in pectinic material are normally available for cross-linkages through multivalent cations, mainly calcium. In transgenic fruits, since SAM precursor is diverted to PA synthesis, it was not readily available for disruption of calcium bridges. The effect of enhanced PAs on maintaining fruit wall rigidity is also ascribable to their action similar to Ca^{2+} in forming cross-linkage to the COO– group of the pectic substances in the cell wall. This cross-linking reportedly blocks the access of degrading enzymes, such as pectin methyl esterase, pectinesterase and PG, reducing the rate of softening during storage (Mirdehghan et al. 2007). Thus, in spite of the enhanced expression of cell wall modifying genes, the

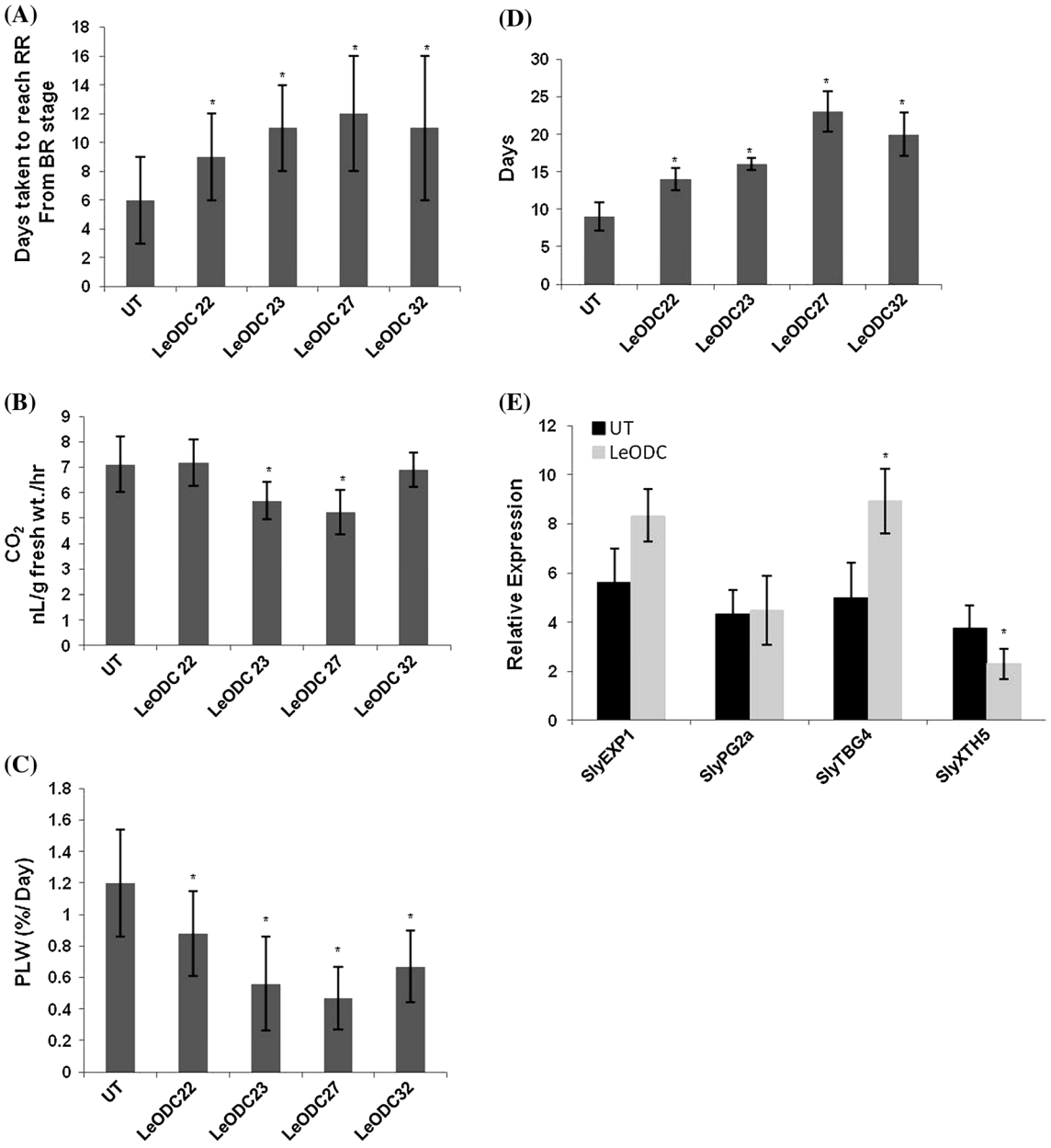

Fig. 4 Keeping qualities of UT and transgenic tomato fruits. Data is the representation of T_3 generation showing means of nine biological replicates in each of the ten independent experiments ($n = 9 \times 10 = 90$). Each set of experiment was repeated in four generations (T_0–T_3) with reproducible results. **a** Delayed on-vine ripening. **b** Respiratory activity in ripening fruits from UT and transgenic plants was measured through gas analyzer. **c** Rate of physiological loss of water was monitored for 10 days in ripe fruits kept at room temperature. **d** Shelf life of ripe fruits kept at room temperature. **e** Relative expression levels of cell wall hydrolyzing genes in UT and LeODC fruits. Relative quantification was performed by qRT-PCR using comparative D cycle threshold (CT) method. Expression levels of all the genes were normalized to SlyActin levels. The data represent the average of three biological replicates, each with two technical replicates and was *plotted with error bars* showing standard deviation. *Significant at $p \geq 0.05$ over UT control. Expression analysis was done in T_3 generation with three biological replicates (sample size $= 3 \times 2$)

increased cellular PAs might be involved in the stabilization of cell wall integrity by reducing cell wall permeability and consequently leading to the lower PLW in transgenic fruits. These results indicate that the increase in PAs during fruit development would decrease the rate of fruit ripening.

Improved nutritional quality in LeODC fruits

Total soluble solids (TSS) content is the key component in determining fruit flavour and juice viscosity and is of special significance for processing industry (Ferguson

Table 1 Fruit quality traits in UT and LeODC lines of tomato

Transgenic line	TSS (°Brix)	TA (g/100 g)	Total sugars (%)	Lycopene (mg/100 g)	AsA (mg/100 g)	Antioxidant (μM Trolox/g)
UT	5.14 ± 0.27	0.37 ± 0.08	1.89 ± 0.12	9.66 ± 0.68	23.22 ± 0.67	1.39 ± 0.0717
LeODC 22	5.19 ± 0.19	0.41 ± 0.11	2.21 ± 0.19*	10.54 ± 0.47	27.51 ± 0.89*	2.011 ± 0.11*
LeODC 23	5.67 ± 0.18*	0.52 ± 0.13*	2.27 ± 0.06*	12.1 ± 0.51*	29.03 ± 0.56*	5.24 ± 0.09*
LeODC 27	6.93 ± 0.31*	0.73 ± 0.10*	2.91 ± 0.14*	14.85 ± 0.34*	36.23 ± 0.78*	4.87 ± 0.185*
LeODC 32	7.12 ± 0.25*	0.67 ± 0.10*	2.26 ± 0.26*	12.78 ± 0.76*	33.35 ± 0.61*	4.63 ± 0.087*

Data is the mean ± standard error, based on at least ten independent experiments with nine fruits in each experiment. Each experiment was replicated in four generations of transgenic lines with reproducible results

* Significant at $p < 0.05$ between UT and LeODC transgenic lines

and Boyd 2002). It comprises of sugars, minerals and acids contents. In the present study, TSS was increased by 30–50 %, in juice of the transgenic fruits as compared to UT fruit juice (Table 1). Transgenic fruits also exhibited ~50 % increase in total sugars and ~2.0 fold increase in titratable acids (TAs) (Table 1). Since organic acids (e.g. citric acid) are the substrates of respiration, which is an ethylene-dependent factor, the lower rate of respiration in transgenic fruits might have resulted in the accumulation of TAs in such fruits (Defilippi et al. 2004).

Comparison of ascorbic acid (AsA) levels between transgenic and UT fruits revealed ~60 % higher levels in fruits of LeODC27 transgenic line over UT fruits (Table 1). AsA is a primary antioxidant and an important phytopharmaceutical found in tomato fruits. Ascorbate functions as a cofactor in ethylene biosynthesis (Watada et al. 1976). AsA levels decline during ripening and senescence, which is correlated with its consumption in ethylene biosynthesis pathway. In present study, the enhanced PA levels might have caused ascorbate accumulation in transgenic fruits by impeding with ethylene biosynthesis. Yahia et al. (2001) also reported the association between the increased cellular concentration of Put with higher ascorbic acid in fruits. High levels of total antioxidants (~fivefold increase) was also recorded in transgenic fruits (Table 1).

The transgenic fruits were visually more dark red in colour than UT fruits, which is linked with the lycopene build-up in such fruits. Transgenic fruits accumulated ~10–40 % more lycopene content than UT fruits (Table 1). The expression pattern of lycopene biosynthesis genes viz., 1-deoxy-D-xylulose 5-phosphate synthase (*DXS1*) and phytoene synthase (*PSY1*) revealed significant increase in their mRNA titres, while transcript profile of lycopene epsilon-cyclase (*LES*) which is a lycopene catabolic gene, did not show any significant difference from UT control (Fig. 5, Supplementary Fig. 5d). Therefore, the up-regulated expression of these transcripts accounts for the enhanced lycopene content in transgenic fruits over UT fruits. The enhanced PA levels might be involved in the increased levels of lycopene and expression of related genes possibly

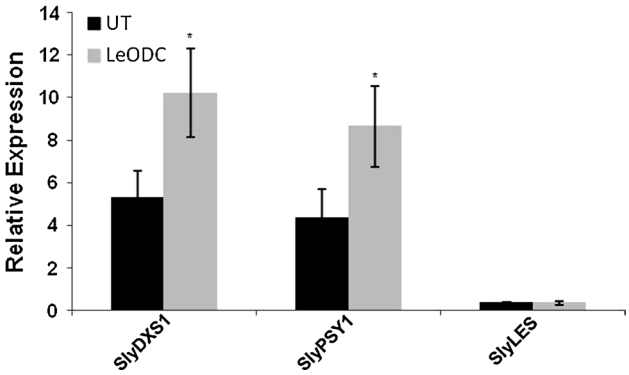

Fig. 5 Expression pattern of lycopene biosynthesis genes in T_3 fruits. Relative quantification was performed by qRT-PCR using comparative D cycle threshold (CT) method. Expression levels of all the genes were normalized to SlyActin levels. The data represent the average of three biological replicates, each with two technical replicates and was *plotted with error bars* showing standard deviation. *Significant at $p \geq 0.05$ over UT control. Expression analysis was done with three biological replicates (sample size = 3 × 2)

by stabilization of mRNA turnover or regulation of lycopene biosynthesis genes. It has been reported that reduced degradation of membrane phospholipids in fruits tend to reduce the turnover of phospholipids and free fatty acids. Since fatty acid biosynthesis and carotenoid biosynthesis share the common precursor (Acetyl CoenzymeA), reduced demand for acetyl CoA for fatty acid biosynthesis in transgenic fruits may tend to enhance their availability for carotene and lycopene biosynthesis (Oke et al. 2003). This could also explain the enhanced lycopene in transgenic fruits. Both these arguments are in agreement with the reports of Mehta et al. (2002), Neily et al. (2011) and Madhulatha et al. (2014) on transgenic tomato with over-expression of yeast *SAMDC* and *SPDSYN* and human *SAMDC* gene. Therefore, PA accumulation in transgenic fruits appears to cause an increase in the phytonutrients in fruits.

In conclusion, the present study proclaims that the PA accumulation in fruits due to the over-expression of a Put

biosynthesis gene, *ODC* can inhibit ethylene emission, reduce the perishability and improve quality of tomato fruits. These results also suggest that Put may regulate fruit ripening in tomato.

Materials and methods

Plant material

The seeds of tomato (*Solanum lycopersicum* Mill. cv. Pusa Ruby) were procured from National Seeds Corporation, New Delhi. Fully expanded cotyledons from 10 to 12 days old tomato seedlings grown in pots containing garden soil and vermiculite (1:1) under controlled growth conditions (26 ± 1 °C, 16 h photoperiod with irradiance of 40 µE mol m^{-2} s^{-1}) were utilized for transformation experiments.

Development of LeODC transgenic tomato plants

A 4.0 kb fruit-specific 2A11 promoter fragment was excised from pGEM-T-2A11 (gift from Dr. V. S. Reddy, International Centre for Genetic Engineering and Biotechnology, New Delhi) by *Sma*I and *Eco*RI restriction digestion, and was ligated in pBinAR binary vector, replacing CaMV 35S promoter. Mouse *ODC* (m-*ODC*) gene (1.7 kb) was fished out from pBSK-ODC (gift from Prof. Tony Pegg, Hershey, USA) plasmid by restriction digestion with *Eco*RI and *Bam*HI enzymes. The m-*ODC* gene fragment was end filled using klenow fragment and was subsequently ligated into *Sma*I site of pBin2A11. The pBin-T-DNA contained sequences encoding the enzyme neomycin phosphotransferase II (*NPT*-II) from the Tn5 transposon of *E. coli* (K12), under the control of NOS promoter from *Agrobacterium tumefaciens* (Supplementary Fig. 1). The resultant binary vector, pBin2A11ODC (Supplementary Fig. 1), was mobilized into *A. tumefaciens* strain LBA4404. The transformed *A. tumefaciens* was used for tomato transformation according to a protocol described by Madhulatha et al. (2007). The expression of *NPT*-II activity was used as a selectable trait to screen transformed plants. Transformed and untransformed (UT) regenerants, with well-developed roots were then transferred to plastic pots and following hardening they were grown in transgenic green-house/net-house for further studies.

Integration and segregation analysis of transgene in tomato transformants

Genomic DNA was isolated from the leaves of UT and tomato transformants using CTAB method (Doyle and Doyle 1990). Utilizing 100 ng of genomic DNA, primary transformants were screened by PCR for the presence of transgene (m-*ODC*) as well as *NPT*-II marker gene. The primer pairs used for the amplification of 750 bp fragment of *NPT*-II gene at 59 °C annealing, were 5′-TCAGAAGAACTCGTCAAGAA-3′ and 5′-ATGGGGATTGAACAAGATGG-3′ and for 570 bp amplicon of m-*ODC* gene at 59 °C annealing, were 5′-ATACCATGGTTTTGGCAGCCATAATGG-3′ and 5′-ATAGGCGCGCCGCTCCTATACCGAACTGA-3′.

Genomic DNA (10 µg), digested with *Xba*I enzyme was used for Southern blot hybridization using radio-labelled m-*ODC* probe. The probe was made using nick translation kit according to manufacturer's guidelines (Bangalore Genei, www.bangloregenei.com/). Blots were prepared using the standard protocol (Sambrook et al. 1989), using nylon membrane (Hybond N, Pharmacia). Pre-hybridization and hybridization were carried out according to Sambrook et al. (1989).

The transgene segregation pattern was scored based on kanamycin resistant phenotype and transgene-specific PCR analysis. A schematic diagram illustrating segregation analysis across different generations has been presented in Supplementary Fig. 3. The leaves from T_1, T_2 and T_3 seedlings (100 seedlings, raised on a 1:1 ratio of soil:vermiculite) of each independent line were surface-sterilized and placed on antibiotic (50 mg/l kanamycin) amended MS basal or shoot regeneration medium to check for the segregation of the transgene based on kanamycin-resistant phenotype. The explants were scored for kanamycin resistance after 15 days of culture. The leaves which survived on antibiotic-amended medium were considered as positive segregants and others that could not survive and died/bleached/leaf yellowing were considered negative segregants and were discarded (Supplementary Fig. 2). Genomic DNA from T_1, T_2 and T_3 positive segregants (forty each) of each transgenic lines along with few UT plants were utilized for segregation analysis studies. Segregation pattern was confirmed by PCR in all the forty progenies from T_1, T_2 and T_3 generations using m-*ODC* specific primers.

Segregation pattern of homozygous lines was also confirmed in T_3 progenies by Southern blot analysis. Genomic DNA (10 µg, digested with *Xba*I enzyme) from three T_3 progenies of few homozygous lines was hybridized with radio-labelled m-*ODC* probe. Preparation of blots and hybridization was carried out as described earlier.

In vitro pollen germination assay

Pollen grains were collected from freshly dehisced anthers of UT and transgenic lines. Pollen were suspended in pollen germination medium (10 % Sucrose, 1,618 µM H_3BO_4, 1,270 µM $Ca(NO_3)_2$, 811 µM $MgSO_4$, 989 µM KNO_3) (Sinha and Rajam 2013) and incubated for 2 h at 26 ± 2 °C in the dark. Pollen germination was recorded under a light microscope. At least 500 pollen grains were counted from

each slide and percentage pollen germination was determined by repeating the experiment thrice.

Determination of different ripening stages in transgenic fruits

Flowers were tagged at anthesis and the days were noted for the fruit formation. Average days from MG (full size and firm) to reach BR and BR to RR were noted for the UT fruit based on the amount of colour each stage fruit will develop (Mehta et al. 2002). The number of average days was then applied for harvesting fruits from transgenic lines, which although now differ in their colour appearance but are at same age as that of their respective controls.

Expression analysis

Pericarp tissue (~100 mg; MG, BR, PR and RR fruits) from three biological replicates was pulverized to homogenate powder. The homogenate was used for isolation of total RNA with the TriZol reagent following manufacturer's guidelines (Invitrogen, http://www.invitogen.com). Total RNA integrity was verified by agarose gel electrophoresis and their purity (A 260/A 280 > 1.95) with a NanoDrop ND-1000 spectrophotometer (Thermofischer, USA). This was followed by RNase free DNase (Fermentas, www.fermentas.com) treatment to remove contaminating genomic DNA. For northern blot analyses, total RNA (15 µg) was fractionated using formaldehyde agarose gel (Sambrook et al. 1989) and blotted onto the positively charged nylon membrane (Hybond N, Pharmacia). The membrane was incubated for 6 h at 42 °C in a prehybridization buffer. Hybridization was carried out using ^{32}P-labeled m-*ODC* and Sly*ACTIN* probes at 42 °C for 16 h (Sambrook et al. 1989).

cDNA was generated from total RNA using Superscript reverse transcriptase (Invitrogen, USA) according to manufacturer's instructions. qRT-PCR was performed using SYBR Green I technology in Realplex2 thermal cycler (Eppendorf, Germany). A master mix was prepared by using MESA Blue qPCR mix as a PCR core reagent (Eurogentec, USA). 1.5 ng of cDNA and 750 nM each of the specific primers were added in a final volume of 10 µl (Table S1). The amplification reactions were carried out at 95 °C 4 min, 40 cycles at 95 °C 15 s followed by 60 °C for 1 min. Specificity of amplification was assessed by disassociation or melt curve analysis at 60–95 °C after 40 cycles. For all the qPCR experiments, three independent biological replicates were included (each with two technical replicates). Relative expression was calculated using comparative D cycle threshold (CT) method and an average of three biological replicates X each with two technical replicates, was plotted along with calculated standard deviation. The constitutively expressed *SlACTIN* gene was used as reference gene for normalization of the expression. Primer pairs used for each gene with their respective accession numbers are provided in Supplementary Table S1.

Initially, semi-Quantitative RT-PCR was also performed to check the transcript level of the few ripening specific genes. DNA-free RNA (50 ng) was used for the one-step RT-PCR reaction as specified in the kit manual (Taurus-Scientific, India, www.taurusscientific.com). The reaction conditions were as follow: 45 °C for 50 min, followed by 94 °C for 5 min, n cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 30 s and finally 72 °C for 15 min. The products were analyzed on ethidium bromide stained 1.5 % agarose gel. All semi-quantitative-RT-PCR experiments were performed in triplicates, with RNA from three fruits.

Accession numbers

The accession numbers for ripening-associated genes selected for transcript analysis are: *SlODC* (NM 001247687.1), *SlADC1* (NM 001247135.1), *SlSAMDC1*(EU196515.1), *SlSPDSYN* (NM 001247564.1), *ACS6* (AF179249), *SlACS1A* (U18056.2), *SlACS2* (NM 001247249.1), *SlACS4* (NM 001247351), *SlACO1* (NM 001247095), *SlE8* (X13437.1), 1-deoxy-D-xylulose-5-phosphate synthase (*SlDXS1*, AF143812), (*SlPSY1*, EF157835.1), lycopene-epsilon cyclase (*SlLES*, Y14387), *SlTAGL1* (AY098735.2), polygalacturonase (*SlPG*, X05656), expansin (*SlEXP1*, U82123), β-galactosidase 4 (*SlTBG4*, AF020390), α-xyloglucan endotransglucosylase/hydrolase (*SlXTH5*, AY497475), and *SlACTIN*, (BT012695).

Quantification of polyamine levels

PAs were estimated by TLC method using 100 mg of tissue each from leaf, floral bud (FB) and pericarp of MG, BR, PR and RR fruits. Tissue was homogenized in 1 mL of 10 % perchloric acid and was centrifuged at 18,000×*g* for 20 min at 4 °C. The resultant supernatant formed the source of free and conjugated fractions of PAs and the pellet as the bound fraction. PA fractions thus obtained, were dansylated, chromatographed and quantified as described by Bajaj and Rajam (1996) using dual wavelength fluorometer (Bio-Rad, Versa Fluor, USA) with an excitation wavelength of 350 nm and emission wavelength of 495 nm. PA analysis was performed in three independent experiments.

Ethylene measurements

In order to determine ethylene levels, three RR fruits (preweighed) were enclosed in 100 mL of air-tight container for about 1 h at room temperature. Headspace atmosphere

(3 mL) of the container was withdrawn and injected into a gas chromatograph (Model HP 5890, Hewlett Packard, USA) (Singh and Pal 2008). The ethylene estimation was carried out in four generations each having ten independent experiments with three experimental replicates.

Estimation of respiration rate

Five pre-weighed RR fruits were sealed in an air-tight container for 1 h and their respiration rate was determined by measuring the head space carbon dioxide (CO_2) concentration using CO_2/O_2 analyzer (Model Checkmate 9900 O_2/CO_2, PBI Dansensor, Denmark) (Singh and Pal 2008). The respiratory activity was measured in ten independent experiments with three experimental replicates (repeated for four generations; T_0–T_3 of transgenic tomato plants). Respiration rate was calculated taking into account the weight of the fruit, residual volume of the container and the incubation time (1 h). CO_2 evolution rate was expressed as mL kg^{-1} h^{-1}.

On-vine ripening period determination

Flowers were tagged at anthesis and days were noted for the fruit formation. Mature fruits displaying first sign of colour change were identified as BR stage. On-vine ripening period was expressed as the number of days taken to reach RR state from BR stage. The experiment was carried out with nine fruits as biological replicates and repeated with ten independent sets. All ten sets were repeated for four generations (T_0–T_3) of transgenic tomato plants.

Determination of fruit shelf life

RR fruits were kept at room temperature and time was noted for the first visual sign of shrivelling after being detached. The shelf life of fruits was noted for nine fruits as biological replicates each in ten independent sets for four generations of transgenic tomato plants.

Determination of physiological loss of water

PLW from tomato plants was determined as loss in weight of tomato fruits. The loss in weight of individual fruit was calculated daily as a percentage of the original weight. A mean of nine fruits in each of the ten independent sampling periods in each generation (repeated for four generations; T_0-T_3 of transgenic tomato plants).

Measurement of total soluble solids

TSS concentration in RR homogenate was measured by a hand refractrometer (Model: Fisher, Japan). Results were expressed as °Brix and were corrected to 20 °C temperature. TSS content was measured in nine fruits as biological replicates in ten independent experiments in each of the four generations; T_0–T_3 of transgenic tomato plants.

Quantification of titratable acids

TAs content was determined by the method of Srivastava and Kumar (1993). A known volume of filtered juice was titrated with 0.1 N NaOH using phenolphthalein indicator and light pink colour as an end point. Acidity was computed and expressed as percent citric acid. The estimation was done in nine biological replicates in each of the ten independent experiments followed for four generations (T_0–T_3) of transgenic tomato plants.

Determination of sugars

Total fruit sugar content was determined following the method of Ranganna (1977). Sugar content was measured in nine fruits as biological replicates in each of the ten independent experiments carried out for four generations (T_0–T_3) of transgenic tomato plants.

Estimation of ascorbic acid content

AsA content in the red fruits was estimated titrimetrically using 2,6-dichlorophenol indophenol as the indicator dye (Singh and Pal 2008). AsA standard was prepared by dissolving 100 mg of L-ascorbic acid in 100 mL of 1 % HPO_3 (Singh and Pal 2008). AsA content was measured in nine fruits in each of the ten independent experiments followed for four generations (T_0–T_3) of transgenic tomato plants.

Quantification of lycopene content

Lycopene fraction of RR fruit pericarp tissue (~2 g) was determined by spectrophotometric method as described by Ranganna (1977). Lycopene fractions were estimated in nine biological replicates in each of the ten independent experiments followed for four generations (T_0–T_3)of transgenic tomato plants.

Determination of total antioxidant activity

The total antioxidant activity was measured in 2 g of fruit pericarp tissue using total radical-trapping antioxidant parameters (TRAP) assay. The total antioxidant extraction and assay was performed according to the procedure reported by Fanasca et al. (2006). The total antioxidant activity was assayed in nine fruits as biological replicates in each of the ten independent experiments carried out for four generations (T_0–T_3) of transgenic tomato plants.

Data collection and analysis

For each of the parameter, data from four consecutive generations, with their replicates was collected. Data are the means of 10 experiments (9 replicates in each experiment). The data presented are the mean with the standard error from all the experiments. Significant differences were determined by Student's *t* test ($p < 0.05$).

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Conflict of interest The authors declare that they have no conflict of interests.

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